Folding and Unfolding of the Tryptophan Zipper in the Presence of Two Thioamide Substitutions

Spekowius, Jasmin ; Pfister, Rolf ; Helbing, Jan

Abstract: We studied the stability and folding and unfolding kinetics of the tryptophan zipper, containing different double thioamide subsitutions. Conformation change was triggered by photoisomerization of an integrated AMPP photoswitch in the turn region of the hairpin, and transient spectra were recorded in the deep UV and the mid-IR, covering the time window of the (un)folding transition from picoseconds to tens of microseconds. Thio-substitution of inward-pointing backbone carbonyls was found to strongly destabilize the -hairpin structures, whereas molecules with two outward pointing thio-carbonyls showed similar or enhanced stability with respect to the unsubstituted sequence, which we attribute to stronger interstrand hydrogen bonding. Thiolation of the two Trp residues closest to the turn can even prevent the opening of the hairpin after cis–trans isomerization of the switch. The circular dichroism due to the two thioamide * transitions is spectrally well-separated from the aromatic tryptophan signal. It changes upon photoswitching, reflecting a local change in coupling and geometry.

DOI: https://doi.org/10.1021/acs.jpcb.1c03327

THE JOURNAL OF PHYSICAL CHEMISTRY B
A JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/JPCB

Article

Folding and Unfolding of the Tryptophan Zipper in the Presence of Two Thioamide Substitutions

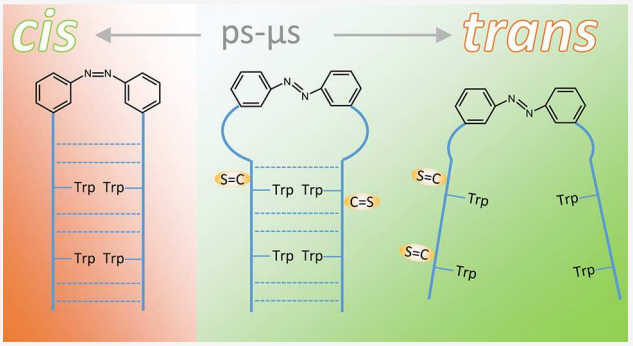
Jasmin Spekowius, Rolf Pfister, and Jan Helbing*

Cite This: *J. Phys. Chem. B* 2021, 125, 7662–7670

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: We studied the stability and folding and unfolding kinetics of the tryptophan zipper, containing different double thioamide subsitutions. Conformation change was triggered by photoisomerization of an integrated AMPP photoswitch in the turn region of the hairpin, and transient spectra were recorded in the deep UV and the mid-IR, covering the time window of the (un)folding transition from picoseconds to tens of microseconds. Thio-substitution of inward-pointing backbone carbonyls was found to strongly destabilize the β-hairpin structures, whereas molecules with two outward pointing thio-carbonyls showed similar or enhanced stability with respect to the unsubstituted sequence, which we attribute to stronger interstrand hydrogen bonding. Thiolation of the two Trp residues closest to the turn can even prevent the opening of the hairpin after cis−trans isomerization of the switch. The circular dichroism due to the two thioamide $\pi\pi^*$ transitions is spectrally well-separated from the aromatic tryptophan signal. It changes upon photoswitching, reflecting a local change in coupling and geometry.



INTRODUCTION

A common method for studying site-specific structure and dynamics of peptides and proteins in solution is to mark selected amino acids with a spectroscopic label. In IR spectroscopy, this can be done almost noninvasively by replacing backbone carbonyl atoms with their ^{13}C and/or ^{18}O isotopes. The C═O stretch vibration (amide I) of the labeled peptide unit is thus red-shifted and isolated from the amide I band of the rest of the peptide or protein near 1650 cm^{-1}. Local dynamics encoded in the $^{13}C═^{18}O$ band can then be interpreted in combination with the information from the $^{12}C═^{16}O$ band of the bulk.[1,2] Inspired by the impact that isotopic labeling had on IR-spectroscopy for resolving site-specific structural changes in peptides,[3,4] we aim to transfer this concept to the UV region, where transition dipoles are larger and structure-sensitive coupling between them is stronger. We try to achieve this by spectroscopic labeling using thioamide substitutions.[5] In thioamides, in which the backbone carbonyl oxygen of the amide is replaced by sulfur, the lowest lying $\pi\pi^*$ absorption band is shifted to 270 nm, far away from the absorption of the oxoamide at 190 nm.[6] When introducing two thioamides in close vicinity into a peptide structure, excitonic coupling is possible, the premise for studying structural changes by specotroscopic methods that are particularly sensitive to coupling such as circular dichroism (CD). Due to the relatively large transition dipole moment of the $\pi\pi^*$ transition, we can expect to create locally structure-sensitive signals in the deep UV, which can be probed by ultrafast broad-band laser pulses.[5]

Although thioamide substitution involves the exchange of only a single atom, it may affect peptide stability, depending on the position of the label within the peptide.[7−11] Thioamide units act as stronger hydrogen bond donors and weaker hydrogen bond acceptors and have been used in studies that investigate the effect of hydrogen bond strength on structure.[12,13] When placed at the center of α-helices, a thioamide unit tends to interrupt the secondary structure[9,10,14] as we could confirm for a number of different sequences (see Supporting Information for examples). This local perturbation has been attributed to steric effects,[9] because the sulfur has a larger van der Waals radius and the C═S bond is slightly longer than the C═O bond in an oxoamide. It can thus be advisable to introduce thioamide labels at positions where the sulfur atom does not introduce a steric perturbation and preferably to substitute outward-pointing, solvent-exposed carbonyls.[11] Even then, however, electronic effects, such as

Received: April 13, 2021
Revised: June 3, 2021
Published: July 7, 2021

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

altered H-bond strengths and perturbation of the backbone n−π^* interaction may not be negligible.[15]

In the current study we have introduced two thioamide labels in a tryptophan-based β-hairpin structure, the so-called tryptophan zipper, and tested different positions in the sequence. The hairpin originally designed by Cochran et al. (TrpZip2) consists of 12 amino acids.[16] In aqueous solution, the peptide folds into a β-hairpin that is stabilized by a hydrophobic core of the Trp side chains in addition to the regular cross-strand hydrogen bridge bonds. It has served as a model peptide to investigate the kinetics and mechanisms of hairpin folding in a number of temperature-jump experiments.[4,17−21] Dong et al.[22] have shown that the β-turn forming amino acids Asn and Gly can be replaced by the photosensitive azobenzene derivative [3-(3-aminomethyl)phenylazo]-phenylacetic acid (abbreviated: AMPP). By this modification, the tryptophan zipper becomes a peptide whose folding state is switchable by light and is thus suitable for time-resolved measurements that are light-triggered. In the thermodynamically favorable trans isomer of the AMPP switch, it is not possible for the two peptide strands to adopt a fully folded hairpin structure. Only when the switch is transferred to its cis conformation by illumination with light of suitable wavelengths can the two strands fold into a β-hairpin. With the AMPP switch in its cis configuration, approximately half of the peptides adopt this hairpin structure in methanol at room temperature.[22] Multiple time-resolved IR studies on the azobenzene-substituted tryptophan zipper in combination with transient electronic spectroscopy and molecular dynamic simulations[23−26] have led to a very detailed picture of its folding and unfolding behavior. It has also been shown that the microsecond folding kinetics triggered by the isomerization of the photoswitch are consistent with the times deduced from temperature-jump unfolding experiments.[25]

In this publication, we build on this knowledge and investigate the suitability of thioamide labels as site-specific reporters of local conformation change in a peptide. In earlier work, Culik et al.[12] have already used single thioamide substitutions to modify the strength of cross-strand hydrogen bridge bonds of the tryptophan zipper variant of Hauser and Keiderling,[4] and they have studied the effect on the folding mechanism by transient IR spectroscopy on the remaining oxoamide units. They observed a significantly faster relaxation kinetics after a temperature-jump when substituting residues at positions 1 and 10. However, they inferred from their equilibrium data that the folding kinetics are unaffected by these modifications of the strength of the interstrand hydrogen bonds, and they concluded that they are formed after the folding transition state.[12] Our sequences (see Figure 1) combine this tryptophan zipper variant with the AMPP switch. Most importantly, they contain not only one, but two thioamide substitutions in order to create site-specific coupling signals in the UV, which can be studied by CD spectroscopy. We could thus selectively trigger both the folding and the unfolding transition, follow the kinetics by time-resolved IR and UV spectroscopy and compare their information content. The chosen positions for the thioamide labels are summarized in Figure 1 and Table 1. The numbering of amino acids follows that of the TrpZip2 sequence without the AMPP turn and 12 amino acids (i.e., AMPP is counted as the two amino acids 6 and 7). In W2s4s (blue) and W4s9s (green), the substituted C═S bonds are directed out of the hairpin structure which should not lead to steric perturbation of the hairpin fold. In addition, the stronger hydrogen bond donating propensity of the thioamide NH may stabilize the native peptide form. In W2s4s, the thioamide labels are located on the same strand so that their coupling should be sensitive to the twisting of this strand. In W4s9s, the two thioamide labels are placed on the two opposing β-strands so that they can report on the opening and closing of the hairpin. In A3s10s (red), the substituted sulfurs point into the hairpin structure where they directly face each other. Due to this vicinity, a strong coupling signal can be expected which should directly report on the state of the hydrophobic Trp side chain core. However, the increased size of the sulfur as well as its weaker hydrogen bond accepting capability may interfere with an intact hairpin structure.

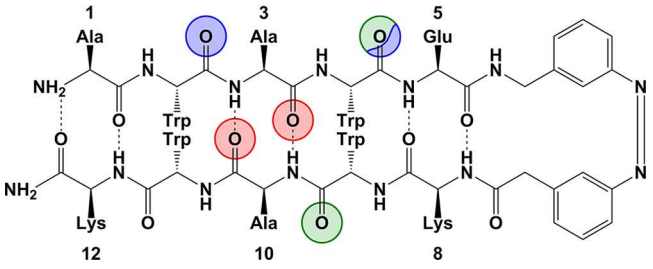


Figure 1. Representation of the photoswitchable tryptophan zipper used in this study. The azobenzene derivative replaces the two amino acids of the turn and allows us to trigger the folding or unfolding of the hairpin by light. The thio-labels are introduced at position 2 and 4 (blue), 4 and 9 (green), and 3 and 10 (red).

Table 1. Sequences of the Azobenzene Tryptophan Zipper and the Three Thioamide-Labeled Variants Including the References Used in This Paper

reference	sequence
oxo	H-AWAWE-AMPP-KWAWK-NH_2
W2s4s	H-AW^SAW^SE-AMPP-KWAWK-NH_2
W4s9s	H-AWAW^SE-AMPP-KW^SAWK-NH_2
A3s10s	H-AWA^SWE-AMPP-KWA^SWK-NH_2

METHODS

Thioamino acid precursors for solid-state synthesis of the substituted peptides were prepared following the protocols in the literature,[13,27] and the AMPP switch was synthesized according to the literature.[22,28] Solid-phase synthesis of the peptides was performed based on the published protocols.[11,29] The peptides were purified via fast protein liquid chromatography, and their purity was confirmed by mass spectrometry. Trifluoric acid from the purification process was removed by dialysis and the samples were lypholized in order to remove all solvent. For spectroscopy, the peptides were dissolved in methanol (deuterated for the IR experiments) and stored up to 1 month in solution until all the experiments were conducted. Methanol is known to promote the hairpin structure and to allow for a concentration of monomeric structures up to the millimolar range.[22] The concentrations were determined by the UV absorption of the Trp side chains, the AMPP photoswitch, and the thioamides. In the steady-state experiments, the optical density was adjusted by varying cuvette thickness between 1 mm and 1 cm, resulting in an approximately 10 μM·cm value for all samples. In the transient UV experiments, the concentrations were around 0.1 mM for the thio-substituted variants and about 0.2 mM for the oxo

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

peptide such that all variants had a similar absorption at 280 nm (1 mm fused silica flow cell in the unfolding experiments, 420 nm excitation, and 2 mm in the folding experiments, 325 nm excitation). In the time-resolved IR experiments, 1.0 mM (oxo), 0.3 mM (W2s4s), 0.7 mM (W4s9s), and 0.5 mM (A3s10s) concentrations were used with a 300 μm CaF_2 flow cell. The yield of the thiopeptide synthesis is rather low (2−7%[11,30]), which is why the concentrations of the thioamide samples were lower than that of the abundant oxoamide sample. In all experiments involving photoswitching (steady state CD and transient spectroscopy), the cis state was accumulated by continuous irradiation at 325 or 340 nm, and the amount of cis structures present in photoequilibrium was calculated from the steady-state absorption at 325 nm, calibrated by ^{1}H NMR experiments, which yielded 80% cis content for all peptide variants. Constant populations of the trans state of the AMPP switch were maintained by continuous irradiation with UV-light at 450 nm, yielding a photostationary state with 80% trans (oxo, W4s9s, A3s10s) and 70% (W2s4s) trans structures.

The time-resolved experiments were conducted in a pump-and-probe manner in which a laser pulse (pump) triggered the isomerization of the AMPP photoswitch, followed by a second laser pulse (probe) in the IR or UV spectral range to record the absorption at different delay times. IR probe pulses were obtained by a home-built two-stage optical parametric amplifier (OPA) .[31] For the transient UV measurements, an in-house setup combining a broadband visible noncollinear OPA[32,33] and an achromatic frequency doubling scheme[34] was used to generate broadband UV probe pulses with spectra ranging from 250 to 320 nm. The fundamental (800 or 840 nm) of the pump pulses was provided by a second, synchronized laser system.[35] Pump pulses of 420 nm wavelength (2−3 μJ per pulse) for triggereing the isomerization of AMPP from cis to trans were obtained by frequency doubling. Pump pulses of 325 nm wavelength (0.4 μJ per pulse) for the opposite trans to cis isomerization were obtained by frequency mixing the 800 nm fundamental with the 550 nm output of another home-built two-stage noncollinear OPA operating in the visible wavelength regime. The pump beam was focused to a spot size of 200−300 μm (fwhm) which was at least two times larger than the focus of the probe (50−100 μm fwhm) to ensure homogeneous excitation of the probed spot. The scanned delay times ranged from 10 ps (jitter of the electronic delay) to 32 or 42 μs (trigger generator). During the time-dependent measurements, the samples were continuously circulated through a flow cell and illuminated with UV light as described above in an external reservoir. All data were acquired at ambient temperature.

RESULTS AND DISCUSSION

Stationary UV absorption spectra of the azobenzene tryptophan zipper and the thio-substituted variants in the trans configuration of the AMPP turn are plotted in Figure 2 (top).

Between 250 and 400 nm, the spectra consist of two distinctive bands: The band peaking at 325 nm is due to the $\pi\pi^*$ transition of the AMPP photoswitch ($\pi\pi^*_{AMPP}$). At 280 nm, the absorption is mainly due to the nπ^* transition of the aromatic side chains of the Trp residues (nπ^*_{Trp}) and two thioamides ($\pi\pi^*_{thio}$) in the thio-substituted derivatives.[36−38] The stationary spectra in Figure 2 were recorded for samples

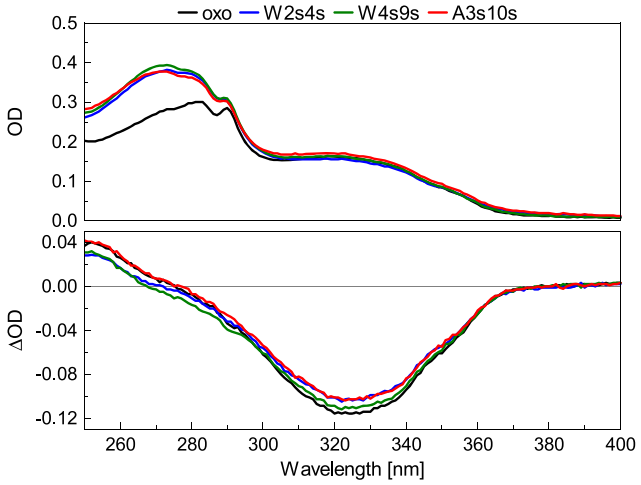


Figure 2. Top: UV absorption spectra of the azobenzene tryptophan zipper and the three thio-substituted variants in the trans configuration of the AMPP photoswitch dissolved in methanol. Bottom: Absorption difference after isomerization of the AMPP switch from trans to a photostationary state of mostly cis isomers. The concentrations were around 10 μM·cm.

that were kept in the dark for more than 12 h, where ^{1}H NMR indicates that more than 90% of the AMPP photoswitches are in trans configuration. Isomerization to cis leads to distinct changes in the UV absorption which are summarized by the difference spectra plotted in Figure 2 (bottom). Upon isomerization from trans to cis, the 325 nm band diminishes and the absorption of the AMPP photoswitch at 250 nm (250_{AMPP}) increases. Near 270 nm, there is an isosbestic point in the absorption spectrum of oxo; i.e., the difference spectrum has a zero-crossing at this wavelength. This can be advantageous, because any absorption changes in this spectral region in the thio-substituted peptides can then be assigned to changes in the thioamide transition dipole coupling. However, when comparing the steady-state differences of oxo and the three thioamide peptide variants, it becomes clear that the absorption changes of the thioamides after isomerization of the turn are only small within those of the AMPP photoswitch.

Because it is intrinsically achiral, AMPP is, however, not contributing to the UV-CD spectra of the four peptide variants, which are plotted in Figure 3 for the two photostationary states of the photoswitch.

The spectra are normalized to concentration and path length of the cuvette (around 10 μM·cm) and number of amino acid residues (10). Upon isomerization of the AMPP moiety, the most prominent spectral changes occur at 228 nm, an excitonic coupling signal of the lowest-lying Trp side chain $\pi\pi^*$ transitions ($\pi\pi^*_{Trp}$) and as such indicative for the hydrophobic core, which is present only in the folded peptide.[39] It is commonly used to estimate the amount of folded hairpin structures: for example, the CD of oxo of 36500 deg cm^2 dmol^{-1} res^{-1} in the cis-photostationary state of the AMPP turn corresponds to 46% folded β-hairpin structures,[16] which is in perfect agreement with the data published by Dong et al. .[22] After isomerization to the trans-photoequilibrium of the AMPP turn, this fraction reduces to 19%. Table 2 summarizes the percentages of folded hairpins of the four peptide variants at the different photostationary isomerization states of the AMPP switch. Note that the fraction of cis isomers present in solution is the same for all peptide variants in the cis-

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

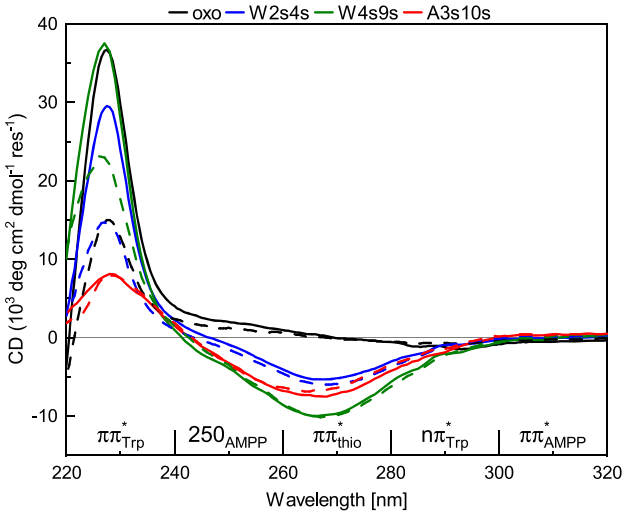


Figure 3. Steady-state UV-CD spectra of the azobenzene tryptophan zipper and the three thioamide substituted variants with the AMPP turn in a photostationary state of predominantly cis (solid lines) or trans isomers (dashed lines).

Table 2. Approximate Fractions of Folded Peptides in the Different Photostationary States (PSS) of the AMPP Turn Calculated from the Steady-State CD Signal at 228 nm and Data Published in the Literature[16 a]

peptide	*cis*-PSS (%)	*trans*-PSS (%)
oxo	46	19
W2s4s	37	18
W4s9s	47	29
A3s10s	10	10

[a]The amount of cis isomers reduces from 80% (*cis*-PSS) to 20% (oxo, W4s9s, A3s10s) or 30% (W2s4s) after isomerization to the *trans*-PSS.

photostationary state, but different in the photoequilibrium of trans-configured turns.

Combining the portion of isomerized turns for oxo (60%) and the decrease of the folded fraction (reduced by 60%) this means that every cis-to-trans isomerization leads to unfolding. In W2s4s, the amount of folded structures in the cis-photostationary state of the AMPP turn is lower than that in oxo. Even though the thioamide substitutions strengthen the hydrogen bridge bonds across the strands of the folded structure, they seem to destabilize the folded state slightly. Just as in oxo, the isomerization yield (50%) is equal to the change of the folded fraction. Interestingly, the folded fraction in W4s9s decreases less (by 40%) than in oxo although the same number of turns are isomerized (60%). This means that only $^2/_3$ of the W4s9s peptides are unfolded by the isomerization of the turn. Even in the trans-photostationary state of the AMPP turn, a comparably large fraction of molecules are folded. It is possible that the stronger cross-strand hydrogen bonds of the thioamide NH stabilize the folded state of W4s9s such that the structure remains folded even with the AMPP turn in its trans configuration. In A3s10s, the amount of folded structures does not depend on the configuration of the AMPP turn and is only of the order of 10%. The formation of the hydrophobic Trp side chain core is apparently hindered by the thioamide substitutions which would be pointing into the hairpin structure. The increased size and weaker hydrogen bonding capability of the sulfur seem to inhibit the native form of this tryptophan zipper variant. In Figure 3, we have also marked the spectral regions of the different UV transitions and nomenclature we are using in this text. Note that the UV-bands are broad and not restricted to the wavelength regions indicated; i.e. they are overlapping but roughly peaking in the center of the marked regions. Further note that the AMPP photoswitch is not contributing significantly to the CD signal, but its transitions are listed here for completeness.

At 270 nm, a negative peak in the CD spectra stems from the thioamide labels introduced in samples W2s4s, W4s9s, and A3s10s, which changes its intensity when the AMPP photoswitch is isomerized. The amplitude changes of the thioamide CD as well as of the oxo control are summarized as difference spectra in Figure 4.

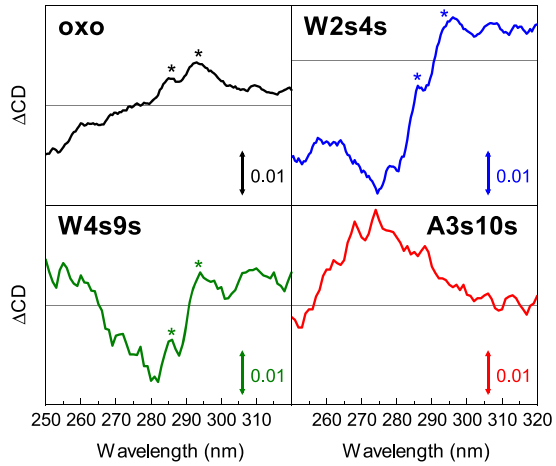


Figure 4. Steady-state CD difference spectra of the azobenzene tryptophan zipper and the three thio-labeled variants upon switching the AMPP moiety from trans to cis (unfolding). Different concentrations were compensated by cell thickness to ≈10 μM·cm for all samples. The arrows correspond to an absorption change of 0.01 mOD or 330 deg cm^2 dmol^{-1} res^{-1}.

The CD difference spectrum of oxo has a zero-crossing point near 270 nm. Any changes at this wavelength in the spectra of the thio-substituted samples can thus be assigned to a contribution of the $\pi\pi^*_{thio}$ transition. The effect of the peptide unfolding on the thioamide CD signal varies between the three different samples: In W2s4s, the CD signal of the thioamide labels is smaller after unfolding. Because the two labels in W2s4s are located on the same strand, this could indicate a change in the bend or twist of this strand affecting the intrinsic chirality of the thioamide unit, but also a change in coupling. Isomerization of the turn in W4s9s changes the thioamide CD signal such that below 265 nm, it increases and above 265 nm, it decreases. In this peptide variant, the two thioamide labels are positioned on opposing strands, and opening of the hairpin is expected to increase their distance and reduce their coupling. In A3s10s, the thioamide CD changes after photoisomerization of the turn even though the peptide does not adopt the native fold in either form. The difference in CD nevertheless indicates a change in the orientation of the two peptide strands with respect to each other or a change in the local environment of the thioamides. In oxo, W2s4s, and W4s9s, the CD difference spectra also show two small peaks near 285 and 295 nm (asterisks in Figure 4), which correspond to the lowest-lying $n\pi^*$ transition of the Trp side chains ($n\pi^*_{Trp}$). This transition

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

only contributes to the CD if the hairpin is folded,[40] in which case the hydrophobic core creates a chiral environment. In the CD difference spectrum of A3s10s, the peaks due to the $n\pi^*_{Trp}$ transition cannot be discerned, which is in line with the observation that also the CD signal of the $\pi\pi^*_{Trp}$ transition at 228 nm remains unaltered in this variant.

In order to further characterize the trans- and cis-ensembles of the four hairpin variants, we recorded time-resolved IR-spectra of the phototriggered unfolding reaction. While the UV-CD spectra are sensitive to the stacking of the Trp side chains and the orientation of the thioamide labels, IR spectroscopy in the region of the amide I band (mainly carbonyl stretch vibration) is sensitive to hydrogen bridge bonds (interstrand or to the solvent) of the peptide backbone. Transient IR absorption changes at different delay times after isomerization of the AMPP turn from cis to trans are plotted in Figure 5.

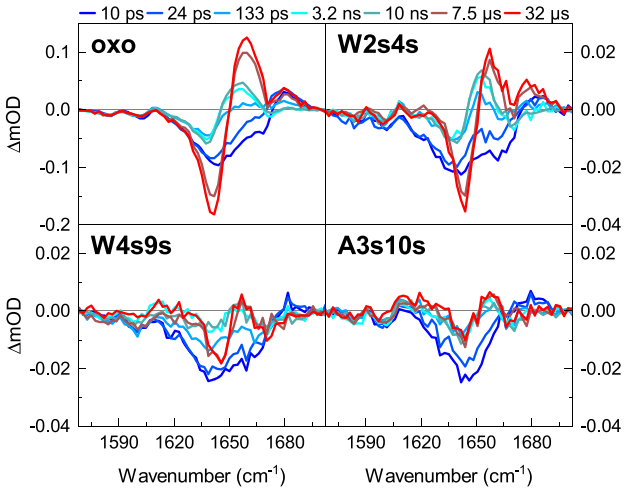


Figure 5. Background-corrected[45] transient IR absorption changes at different delay times after triggering the unfolding of the azobenzene tryptophan zipper and the three thio-labeled variants.

The spectra of the oxo sample agree with the results in the literature[23,24,26] and have been interpreted by these authors as follows: Two small bands at 1585 and 1600 cm^{-1} are due to the AMPP photoswitch in its original cis state and are therefore bleached immediately after excitation and at all subsequent delay times. The 10 ps spectrum also shows a (slightly red-shifted) bleach of the amide I band (1640−1675 cm^{-1}) with an enhanced absorption decrease at 1640 cm^{-1}, which was attributed to the breaking of strong cross-strand hydrogen bonds, and a positive contribution at 1680 cm^{-1}, assigned to the exposure of originally hydrogen bonded carbonyls to the solvent. Both features have been associated with the opening of the turn region and rearrangement of the amino acids in direct vicinity of the photoswitch. Here, 130 ps after excitation, the negative 1675 cm^{-1} shoulder and the positive contribution at 1680 cm^{-1} have disappeared and only the bleach at 1640 cm^{-1} is still present, which has been interpreted as the breaking of additional hydrogen bonds close to the turn. After 3 ns, a positive contribution at 1660 cm^{-1} has grown in. At this time, all hydrogen bonds close to the photoswitch are believed to be broken, while the hydrophobic Trp side chain cluster is still intact and hydrogen bonds here are still present. On a time scale of several microseconds, the amplitude of the difference spectrum grows by a factor of 2 and its zero-crossing red-shifts until the transient absorption spectrum closely resembles the steady-state difference spectrum.[24] During this time, the remaining hydrogen bonds of the hairpin are believed to open, followed by the breaking of the hydrophobic cluster and diffusion of the strands into an ensemble of unfolded structures.

The transient IR response of W2s4s closely resembles that of oxo, only with a lower amplitude due to the lower concentration of the sample, and with slightly altered transition times. The positive signal at 1660 cm^{-1} seems to rise a little earlier in W2s4s than in oxo (compare spectra at 133 ps), but given the similarity of the transient IR spectra, it can be concluded that they follow similar unfolding pathways. It should be remembered, however, that two amide I chromophores are missing from the thio-substituted variants. For W4s9s and A3s10s, on the other hand, the transient IR spectra are only initially similar to those of oxo and W2s4s, but very different at long delay times. In W4s9s, the only spectral change observed after 3 ns consist of a slight growth of the bleach at 1640 cm^{-1}, which may indicate the breaking of a few strong hydrogen bonds. The transient signal at the longest delays closely resembles the steady-state IR difference spectrum (but for a small positive contribution at 1680 cm^{-1}, see the Supporting Information); i.e., the unfolding transition is essentially finished 30 μs after the isomerization of the turn. Nevertheless, relative to the initial bleach, the final spectrum of W4s9s has only half the amplitude of that of oxo and W2s4s. This is in line with the UV-CD data (Figure 3), which show that far fewer structures are unfolded by the isomerization of AMPP. It indicates that the hydrophobic core with thio-substitutions at positions 4 and 9 can better resists the strain exerted by AMPP moiety in the trans state, possibly due to the strengthening of two adjacent interstrand hydrogen bonds. For A3s10s, the transient spectra remain constant after 3 ns. In this sample, the strongly hydrogen-bonding, inward-pointing carbonyls, whose frequency and oscillator strength is expected to change most upon unfolding, have been replaced by sulfur and thus do not contribute to the IR signal.[12] The opening of the hydrophobic core, which takes place on a microsecond time scale in the other samples, may therefore not be visible in the amide I band of A3s10s. In addition, the CD spectra of this variant suggest that the peptide does not adopt a folded hairpin structure even with the AMPP turn in its cis configuration, and it is most likely that no or only a few cross-strand hydrogen bonds are present in this peptide. Overall, the findings of transient IR data of all four peptides, which reveal differences in hydrogen bonding, agree well with the results of the steady-state UV-CD measurements, which record changes of the hydrophobic core.

The main motivation of this study was to test the suitability of thioamides as site-sensitive backbone UV-labels. We thus recorded broad-band transient UV spectra between 250 and 320 nm after triggering the unfolding reaction (cis to trans isomerization of AMPP) of the tryptophan zipper and the thio-substituted variants. To the best of our knowledge, time-resolved UV spectra have not yet been recorded for this or a comparable sequence. The absorption changes of the isomerized AMPP photoswitch dominate the signal at all delays and are similar to the steady-state differences in Figure 2 already after 10 ps. Thus, in order to identify smaller contributions more easily, the transient spectrum at 42 μs was subtracted from the data set presented in Figure 6.

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

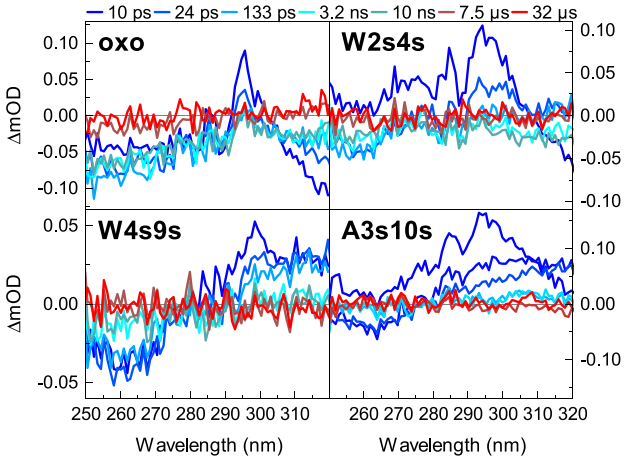


Figure 6. Transient UV absorption changes at different delay times after triggering the unfolding of the azobenzene tryptophan zipper and the three thio-labeled variants. The final spectra at 42 μs have been subtracted from each data set. See the Supporting Information for raw data.

In all four peptides, distinct signals in the $n\pi^*_{Trp}$ region (290−300 nm) can be seen at early delays (blue). They are typical for heating and subsequent cooling of the molecule after photo excitation, which leads to a transient shift or broadening of the absorption spectrum, enhancing its most structured features. Of the thiopeptide variants, the transient spectra of W2s4s resemble those of oxo the most, but slight differences can be discerned near 270 nm. These might indicate a shift of the thioamide absorption, which could, however, not be clearly confirmed by our further analysis below. This also applies to a possible negative thioamide band at 260 nm in the spectra of W4s9s and A3s10s.

Cuts through Figure 6 in the thioamide absorption region at 260 nm are plotted in Figure 7 in combination with the results of the global fit of the full data set (see below). For comparison, crosses and dashed lines show the corresponding IR changes at the maximum of the bleach at 1642 cm^{-1}.

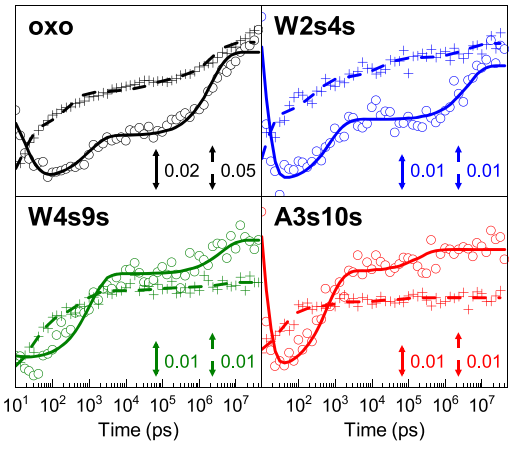


Figure 7. Time-dependence of the transient absorption at 260 nm of the azobenzene tryptophan zipper and the three thio-labeled variants after triggering the unfolding reaction. Open circles represent the measured data points, and the solid lines are the result of a global fit of three exponential decay functions. For comparison, crosses and dashed lines show the corresponding changes in the IR at 1642 cm^{-1}. Vertical scale bars are given in mOD.

In all peptide variants, the kinetics at 260 nm follow the same qualitative behavior: photoisomerization of AMPP photoswitch leads a fast increase of the bleach signal on a 10 ps time scale, largely determined by our time-resolution (jitter between the two laser systems). Subsequently the bleach decreases in two distinct steps with ≈1 ns and 2−4 μs time constants, before reaching the final value (given by the subtracted signal after 42 μs). However, the amplitudes of the two decays vary between the different peptide variants. In oxo and W2s4s, about half of the absorption changes take place with the intermediate ns time constant. In W4s9s, on the other hand, about three-quarters of the signal changes already occur during this intermediate step. Just like in the IR measurements, the contribution from microsecond kinetics is thus much smaller than for oxo and W2s4s. This corroborates our interpretation that AMPP isomerization in W4s9s only leads to a partial unfolding of the strand due to stabilization by two adjacent stronger interstrand hydrogen bonds. For A3s10s, based on the time-resolved IR results, we are not expecting significant spectral changes on time scales slower than nanoseconds. Indeed, the very small second step might be caused by a shock wave in the sample. It is present in all the kinetic traces and we found it impossible to correct as we could for the transient IR spectra. The small changes of the A3s10s signal on the microsecond time-scale in the UV also show that similarly small changes in the transient IR measurements are not due to the absence of the inward-pointing amide I oscillators but rather to the very small change in (mis)folding as reported by the UV-CD data. It is important to note that the 260 nm data points in Figure 7 do not deviate significantly from the lines representing the global fits over the complete spectral range, and the results are surprisingly similar for oxo and W2s4s, despite the additional absorption of the two thio-labels at this wavelength (see Figure 2). Distinct signals of the thioamide units could thus only be observed in the UV-CD signals (Figure 3) but not in the transient or steady-state UV-absorption, where the contributions of the AMPP turn and the thioamides are apparently not separable.

This is further borne out by the decay-associated spectra (DAS) from the global fit. A minimum of three time constants, which are all summarized in Table 3, are necessary to describe the collected UV transients (for details, see the Supporting Infomation).

Table 3. Global Time Constants of the Transient UV Absorption Changes after Triggering the Unfolding Reaction (Cis to Trans) of the Azobenzene Tryptophan Zipper and Thio-Substituted Variants

	oxo	W2s4s	W4s9s	A3s10s
ultrafast	17 ps	7 ps	10 ps	6 ps
intermediate	1.0 ns	740 ps	1.1 ns	620 ps
slow	2.2 μs	4.1 μs	3.8 μs	0.2 μs

The spectra (DAS) associated with the intermediate and slow decay time constants are plotted in Figure 8.

On the nanosecond time scale, the DAS values of all samples show a negative contribution in the region of the 250 $_{AMPP}$ transition, while a positive change of the $\pi\pi^*_{AMPP}$ transition is clearly seen for the thiopeptides only. Due to the similarity of the thio- and oxopeptide DAS in the region of the thioamide absorption, possible contributions from the $\pi\pi^*_{thio}$ transition

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

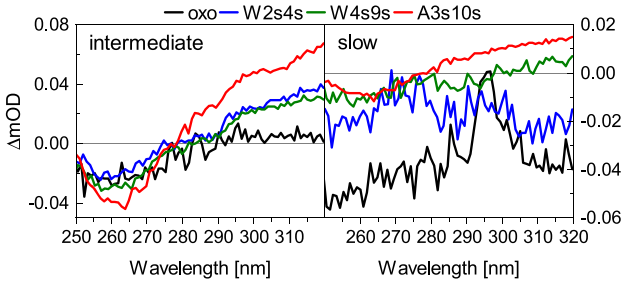


Figure 8. Decay-associated spectra of the unfolding reaction of the azobenzene tryptophan zipper and thio variants associated with the intermediate (≈1 ns) and slow (2−4 μs) unfolding time.

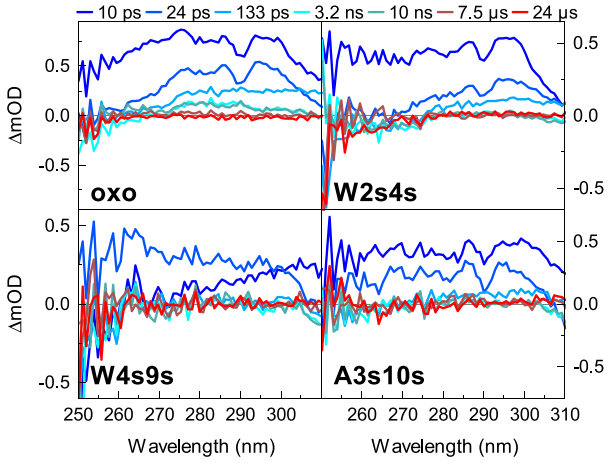


Figure 9. Transient UV absorption changes at different delay times after triggering the folding of the azobenzene tryptophan zipper and the three thio-labeled variants. The final spectra at 32 μs have been subtracted from each data set. See the Supporting Information for raw data.

can unfortunately not be distinguished from those of 250 $_{AMPP}$. Most interestingly, a characteristic $n\pi^*_{Trp}$ signal near 290 nm is not only seen on the picosecond time scale (blue lines in Figure 6), where it can be attributed to the heating and cooling of the molecule after excitation, but also is clearly visible in the microsecond DAS of oxo and W2s4s and very slightly also W4s9s. This indicates significant rearrangement of the Trp side chains in parallel with the breaking of the interstrand hydrogen bonds. Note that the $n\pi^*_{Trp}$ band also contributes slightly to the DAS of oxo, W2s4s, and W4s9s with a nanosecond time constant. Apparently, the hydrophobic core already senses the early conformation changes, possibly via the strain, which the isomerized AMPP switch induces on the strands. In turn, the AMPP moiety is sensitive to the unfolding of the entire tryptophan zipper, as indicated by the microsecond kinetics observed at 260 nm.

Compared to the kinetics observed in the C═O stretch region, the UV transients are much more exponential and the initial heat signal is better separated from the intermediate nanosecond step in Figure 7. Heat dissipation to the solvent is well-known to lead to spectral changes in transient vibrational spectra with apparently nonexponential kinetics on time scales up to hundreds of picoseconds.[41] In addition, excess heat can strongly weaken the amide I oscillator strength of hydrogen-bonded molecules long before significant structural rearrangement.[42] Up to delays of 2−3 ns, we observe very similar spectral changes and kinetics for unfolding, partially unfolding and misfolded sequences both in the UV and the C═O stretch spectral window. This leads us to conclude that these early signals reflect rather generically the thermal and structural relaxation in the vicinity of a constrained AMPP moiety after photoisomerization. On the microsecond time scale, our data does not allow us to distinguish the two time constants (2 and 16 μs) reported in earlier work,[26] which could measure out to longer delay times, and our exponential fits probably average over the two (Figure 7). Surprisingly, however, the stronger interstrand hydrogen bonds expected for the substitution of outward-pointing carbonyls do not seem to slow down the unfolding kinetics significantly, despite the fact that they do prevent a subensemble of the W4s9s variant from unfolding altogether.

Finally, we also recorded transient UV spectra for the folding transition, induced by triggereing the trans to cis isomerization of AMPP at 325 nm. They are equally dominated by the AMPP signal, and Figure 9 again shows the data with the longest delay spectrum subtracted to enhance smaller changes.

Deviations from the final spectra are essentially limited to the subnanosecond time scale. Most prominently, the heat signal with the structured the $n\pi^*_{Trp}$ feature dominates at the early delay times. Its relative size appears to be larger than in the unfolding direction, which may be explained by the greater excess energy and contribution from molecules, which do not isomerize, due to the smaller trans to cis quantum efficiency. According to earlier transient IR-studies[23,25] the main folding step of the azobenzene tryptophan zipper takes place on a 10−30 μs time scale. However, there is no clear signature of this transition in our transient UV spectra, nor is there any unique signal due to the thioamide aborption. Only with W2s4s, we see a faint hint of an absorption change on the microsecond time scale in the region of the $\pi\pi^*_{thio}$ band near 260 nm. The AMPP switch, which dominates the transient UV-signal, is sensitive to conformation changes in the rest of the peptide mainly via strain. Small spectral changes on the time scale of a few hundred picoseconds (see decay-associated spectra in the Supporting Information) may thus be attributed to local relaxation in the vicinity of the switch. However, according to our CD data, a significant fraction of the unfolded molecules does not fold into a zipper, even after trans to cis isomerization, while every cis to trans isomerization does seem to induce unfolding of the oxo and W2s4s zippers. Relative to the subnanosecond signals, UV spectral changes linked to the formation of the hydrophobic core may thus be much smaller. The $n\pi^*_{Trp}$ signatures that indicate the opening of the hydrophobic core several microseconds after triggering unfolding may thus simply escape detection in the folding direction at the end of our measured time window.

SUMMARY AND CONCLUSION

The original goal of this work was to test the suitability of thioamide substitution for the site-sensitive probing of peptide backbone conformation and dynamics by broad-band UV-spectroscopy. We chose the tryptophan zipper with an integrated AMPP photoswitch as a well-studied β-hairpin model and replaced two backbone carbonyls by thiocarbonyls at different positions of the molecule in order to create two coupled transition dipoles, which are spectrally isolated from the other backbone transitions. Both folding and unfolding reactions were triggered by photo isomerization of AMPP, and

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670

transient UV and IR spectra were compared to those of the unsubstituted ("oxo") sequence. In combination with the CD signal of the Trp residues in the two isomerization states of AMPP, this allowed us to draw the following conclusions about the stability of the thio-substituted azobenzene tryptophan zippers: When the two thioamide labels are placed on the same strand and the sulfurs are pointing out of the folded hairpin (W2s4s), the stability of the folded structures is only slightly reduced, but the unfolding mechanism appears to be identical with oxo. When the thioamide labels are placed on opposing strands and the sulfurs are facing away from the hairpin core (W4s9s), the folded structure becomes more stable. Even in the trans configuration of the turn, we found a significant amount of folded hairpins, and cis−trans isomerization only leads to the unfolding of a fraction of molecules. When the thioamide is placed on opposing strands at the center of the structure, such that the sulfurs would be pointing toward each other (A3s10s), the native fold is strongly perturbed. Apparently, the large sulfur radius and reduced interstrand hydrogen bond strengths prevent this peptide from adopting the hairpin structure. Despite these significant differences, the transient UV and IR spectra of all four sequences were found to be surprisingly similar during the first nanosecond after AMPP excitation, indicating that most changes observed on these time scales reflect heat dissipation and relaxation of a constrained AMPP moiety. On the other hand, only for the two properly unfolding sequences (oxo and W2s4s) the $n\pi^*_{Trp}$ absorption in the UV near 290 nm was found to change on a microsecond time scale. It may thus serve as an independent observable for the opening of the hydrophobic core, which appears to start in parallel with the breaking of the interstrand hydrogen bonds. Unfortunately, the absorption changes of the thioamides in the 260 nm region turned out to be very small and could not be clearly distinguished from the overlapping, much larger spectral changes due to the photoswitch. Nevertheless, their CD-signal can be clearly separated from those of the intrinsically achiral switch and the Trp side chains. Furthermore, coupling changes of the two thioamide $\pi\pi^*$ transition dipoles upon AMPP isomerization are apparent from our steady-state CD difference measurements. This is a significant step forward with respect to isotope labeling in the IR, where the coupling of two carbonyls would be prohibitively small at these distances. In a similar spirit, Mukherjee and Gai[43] have demonstrated that excitonic coupling of nitrile-derivatized aromatic side chains gives rise to spectrally well-separated CD-couplets in the 230−250 nm spectral window. We have originally designed the thio-labeled zipper molecules for time-resolved broadband-UV CD spectroscopy,[5] which is currently limited to wavelengths above 250 nm, although we were expecting some site-specific signal already in transient absorption. These very weak UV absorption changes of thioamides strongly contrast with the high sensitivity of the carbonyl stretch frequency and oscillator strength to smallest variations in temperature and environment. At the same time, this sensitivity of the single oscillators in the IR often make it difficult to single out changes in coupling between oscillators.[44] The electronic transition dipole moments of the thioamide labels are much less perturbed by their immediate environment, and they may therefore serve as more specific reporters on local coupling changes in the future. We are currently improving our transient UV-CD setup in order to reach the required sensitivity and to cover all relevant time scales.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpcb.1c03327.

CD of thio-substiuted α-helical peptides, mass spectra of purified hairpin samples, calibration of cis and trans content, raw data, and details of the global fitting (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Jan Helbing − *Department of Chemistry, University of Zurich, CH-8057 Zürich, Switzerland;* orcid.org/0000-0003-3911-8519; Email: jan.helbing@chem.uzh.ch

Authors

Jasmin Spekowius − *Department of Chemistry, University of Zurich, CH-8057 Zürich, Switzerland;* orcid.org/0000-0002-5737-8659

Rolf Pfister − *Department of Chemistry, University of Zurich, CH-8057 Zürich, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jpcb.1c03327

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Swiss National Science Foundation (200021_169742 and 200020_192240) and the Alfred-Werner Legacy of the University of Zurich. We thank Tim Keiderling for very helpful discussions about TrpZip design and stability.

■ REFERENCES

(1) Fang, C.; Wang, J.; Charnley, A. K.; Barber-Armstrong, W.; Smith, A. B.; Decatur, S. M.; Hochstrasser, R. M. Two-dimensional infrared measurements of the coupling between amide modes of an alpha-helix. *Chem. Phys. Lett.* **2003**, *382*, 586−592.

(2) Woys, A. M.; Almeida, A. M.; Wang, L.; Chiu, C. C.; McGovern, M.; De Pablo, J. J.; Skinner, J. L.; Gellman, S. H.; Zanni, M. T. Parallel β-sheet vibrational couplings revealed by 2D IR spectroscopy of an isotopically labeled macrocycle: Quantitative benchmark for the interpretation of amyloid and protein infrared spectra. *J. Am. Chem. Soc.* **2012**, *134*, 19118−19128.

(3) Ihalainen, J. A.; Paoli, B.; Muff, S.; Backus, E. H. G.; Bredenbeck, J.; Woolley, G. A.; Caflisch, A.; Hamm, P. Alpha-Helix folding in the presence of structural constraints. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 9588−9593.

(4) Hauser, K.; Krejtschi, C.; Huang, R.; Wu, L.; Keiderling, T. A. Site-specific relaxation kinetics of a tryptophan zipper hairpin peptide using temperature-jump IR spectroscopy and isotopic labeling. *J. Am. Chem. Soc.* **2008**, *130*, 2984−2992.

(5) Oppermann, M.; Spekowius, J.; Bauer, B.; Pfister, R.; Chergui, M.; Helbing, J. Broad-Band Ultraviolet CD Spectroscopy of Ultrafast Peptide Backbone Conformational Dynamics. *J. Phys. Chem. Lett.* **2019**, *10*, 2700−2705.

(6) Hollósi, M.; Kollát, E.; Kajtár, J.; Kajtár, M.; Fasman, G. D. Chiroptical labeling of folded polypeptide conformations: The thioamide probe. *Biopolymers* **1990**, *30*, 1061−1072.

(7) Miwa, J. H.; Patel, A. K.; Vivatrat, N.; Popek, S. M.; Meyer, A. M. Compatibility of the thioamide functional group with β-sheet

secondary structure: Incorporation of a thioamide linkage into a β-hairpin peptide. *Org. Lett.* **2001**, *3*, 3373−3375.
(8) Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E. Conformational stability of helical peptides containing a thioamide linkage. *Org. Lett.* **2002**, *4*, 4655−4657.
(9) Reiner, A.; Wildemann, D.; Fischer, G.; Kiefhaber, T. Effect of thioxopeptide bonds on α-helix structure and stability. *J. Am. Chem. Soc.* **2008**, *130*, 8079−8084.
(10) Nelli, Y. R.; Antunes, S.; Salaün, A.; Thinon, E.; Massip, S.; Kauffmann, B.; Douat, C.; Guichard, G. Isosteric Substitutions of Urea to Thiourea and Selenourea in Aliphatic Oligourea Foldamers: Site-Specific Perturbation of the Helix Geometry. *Chem. - Eur. J.* **2015**, *21*, 2870−2880.
(11) Walters, C. R.; Szantai-Kis, D. M.; Zhang, Y.; Reinert, Z. E.; Horne, W. S.; Chenoweth, D. M.; Petersson, E. J. The effects of thioamide backbone substitution on protein stability: a study in α-helical, β-sheet, and polyproline II helical contexts. *Chem. Sci.* **2017**, *8*, 2868−2877.
(12) Culik, R. M.; Jo, H.; Degrado, W. F.; Gai, F. Using thioamides to site-specifically interrogate the dynamics of hydrogen bond formation in β-sheet folding. *J. Am. Chem. Soc.* **2012**, *134*, 8026−8029.
(13) Newberry, R. W.; Vanveller, B.; Guzei, I. A.; Raines, R. T. N → π* interactions of amides and thioamides: Implications for protein stability. *J. Am. Chem. Soc.* **2013**, *135*, 7843−7846.
(14) Tran, T. T.; Zeng, J.; Treutlein, H.; Burgess, A. W. Effects of thioamide substitutions on the conformation and stability of α- and 310-helices. *J. Am. Chem. Soc.* **2002**, *124*, 5222−5230.
(15) Newberry, R. W.; VanVeller, B.; Raines, R. T. Thioamides in the collagen triple helix. *Chem. Commun.* **2015**, *51*, 9624−9627.
(16) Cochran, A. G.; Skelton, N. J.; Starovasnik, M. A. Tryptophan zippers: Stable, monomeric β-hairpins. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 5578−5583.
(17) Snow, C. D.; Qiu, L.; Du, D.; Gai, F.; Hagen, S. J.; Pande, V. S. Trp zipper folding kinetics by molecular dynamics and temperature-jump spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 4077−4082.
(18) Jones, K. C.; Peng, C. S.; Tokmakoff, A. Folding of a heterogeneous β-hairpin peptide from temperature-jump 2D IR spectroscopy. *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 2828−2833.
(19) Lin, C. W.; Culik, R. M.; Gai, F. Using VIP T-jump to distinguish between different folding mechanisms: Application to BBL and a Trpzip. *J. Am. Chem. Soc.* **2013**, *135*, 7668−7673.
(20) Yang, W. Y.; Gruebele, M. Detection-dependent kinetics as a probe of folding landscape microstructure. *J. Am. Chem. Soc.* **2004**, *126*, 7758−7759.
(21) Wang, T.; Xu, Y.; Du, D.; Gai, F. Determining β-sheet stability by fourier transform infrared difference spectra. *Biopolymers* **2004**, *75*, 163−172.
(22) Dong, S. L.; Löweneck, M.; Schrader, T. E.; Schreier, W. J.; Zinth, W.; Moroder, L.; Renner, C. A photocontrolled β-hairpin peptide. *Chem. - Eur. J.* **2006**, *12*, 1114−1120.
(23) Schrader, T. E.; Schreier, W. J.; Cordes, T.; Koller, F. O.; Babitzki, G.; Denschlag, R.; Renner, C.; Löweneck, M.; Dong, S. L.; Moroder, L.; et al. Light-triggered β-hairpin folding and unfolding. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 15729−15734.
(24) Schrader, T. E.; Cordes, T.; Schreier, W. J.; Koller, F. O.; Dong, S. L.; Moroder, L.; Zinth, W. Folding and unfolding of light-triggered β-hairpin model peptides. *J. Phys. Chem. B* **2011**, *115*, 5219−5226.
(25) Deeg, A. A.; Rampp, M. S.; Popp, A.; Pilles, B. M.; Schrader, T. E.; Moroder, L.; Hauser, K.; Zinth, W. Isomerization- and temperature-jump-induced dynamics of a photoswitchable β-hairpin. *Chem. - Eur. J.* **2014**, *20*, 694−703.
(26) Rampp, M. S.; Hofmann, S. M.; Podewin, T.; Hoffmann-Röder, A.; Moroder, L.; Zinth, W. Time-resolved infrared studies of the unfolding of a light triggered β-hairpin peptide. *Chem. Phys.* **2018**, *512*, 116−121.
(27) Mukherjee, S.; Verma, H.; Chatterjee, J. Efficient Site-Specific Incorporation of Thioamides into Peptides on a Solid Support. *Org. Lett.* **2015**, *17*, 3150−3153.
(28) Aemissegger, A.; Kräutler, V.; van Gunsteren, W. F.; Hilvert, D. A Photoinducible β-Hairpin. *J. Am. Chem. Soc.* **2005**, *127*, 2929−2936.
(29) Walters, C. R.; Ferrie, J. J.; Petersson, E. J. Dithioamide substitutions in proteins: Effects on thermostability, peptide binding, and fluorescence quenching in calmodulin. *Chem. Commun.* **2018**, *54*, 1766−1769.
(30) Walters, C. R.; Ferrie, J. J.; Petersson, E. J. Dithioamide substitutions in proteins: Effects on thermostability, peptide binding, and fluorescence quenching in calmodulin. *Chem. Commun.* **2018**, *54*, 1766−1769.
(31) Hamm, P.; Kaindl, R. A.; Stenger, J. Noise suppression in femtosecond mid-infrared light sources. *Opt. Lett.* **2000**, *25*, 1798.
(32) Cerullo, G.; De Silvestri, S. Ultrafast optical parametric amplifiers. *Rev. Sci. Instrum.* **2003**, *74*, 1−18.
(33) Manzoni, C.; Cerullo, G. Design criteria for ultrafast optical parametric amplifiers. *J. Opt. (Bristol, U. K.)* **2016**, *18*, 103501.
(34) Baum, P.; Lochbrunner, S.; Riedle, E. Tunable sub-10-fs ultraviolet pulses generated by achromatic frequency doubling. *Opt. Lett.* **2004**, *29*, 1686.
(35) Bredenbeck, J.; Helbing, J.; Hamm, P. Continuous scanning from picoseconds to microseconds in time resolved linear and nonlinear spectroscopy. *Rev. Sci. Instrum.* **2004**, *75*, 4462−4466.
(36) Gill, S. C.; Von Hippel, P. H. Calculation of protein extinction coefficients from amino acid sequence. *Anal. Biochem.* **1989**, *182*, 319−326.
(37) Helbing, J.; Bregy, H.; Bredenbeck, J.; Pfister, R.; Hamm, P.; Huber, R.; Wachtveitl, J.; De Vico, L.; Olivucci, M. A fast photoswitch for minimally perturbed peptides: Investigation of the trans → cis photoisomerization of N-methylthioacetamide. *J. Am. Chem. Soc.* **2004**, *126*, 8823−8834.
(38) Sifferlen, T.; Rueping, M.; Gademann, K.; Jaun, B.; Seebach, D. beta-Thiopeptides: Synthesis, NMR solution structure, CD spectra, and photochemistry. *Helv. Chim. Acta* **1999**, *82*, 2067−2093.
(39) Grishina, I. B.; Woody, R. W. Contributions of tryptophan side chains to the circular dichroism of globular proteins: Exciton couplets and coupled oscillators. *Faraday Discuss.* **1994**, *99*, 245−262.
(40) Huang, R.; Wu, L.; McElheny, D.; Bouř, P.; Roy, A.; Keiderling, T. A. Cross-strand coupling and site-specific unfolding thermodynamics of a trpzip β-Hairpin Peptide using 13C isotopic labeling and IR spectroscopy. *J. Phys. Chem. B* **2009**, *113*, 5661−5674.
(41) Hamm, P.; Ohline, S. M.; Zinth, W. Vibrational cooling after ultrafast photoisomerization of azobenzene measured by femtosecond infrared spectroscopy. *J. Chem. Phys.* **1997**, *106*, 519−529.
(42) Cervetto, V.; Pfister, R.; Helbing, J. Time-resolved infrared spectroscopy of thiopeptide isomerization and hydrogen-bond breaking. *J. Phys. Chem. B* **2008**, *112*, 3540−3544.
(43) Mukherjee, D.; Gai, F. Exciton circular dichroism couplet arising from nitrile-derivatized aromatic residues as a structural probe of proteins. *Anal. Biochem.* **2016**, *507*, 74−78.
(44) Cervetto, V.; Hamm, P.; Helbing, J. Transient 2D-IR Spectroscopy of Thiopeptide Isomerization. *J. Phys. Chem. B* **2008**, *112*, 8398−8405.
(45) Baseline offsets and tilts, related to the time-dependent heating ($\delta T \leq 0.05$ K) and relaxation of the solvent (thermal lensing/shock wave) were corrected by subtracting a linear function setting the end points of the spectral window to zero (see Supporting Information for raw data).

https://doi.org/10.1021/acs.jpcb.1c03327
J. Phys. Chem. B 2021, 125, 7662−7670